ON UROBILIN. PART II. THE PER-CENTAGE COMPOSITION OF UROBILIN.

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VII. *The Hydrobilirubin of Maly.*

It was originally our intention to have commenced this second part of our paper with a discussion of the various artificial products resembling urobilin which have been obtained by the action of chemical reagents upon bilirubin and hæmatin, but the study of these products proved of such complexity and the work expanded so much as it progressed that we find ourselves compelled to relegate the discussion of this subject to yet a further instalment and to content ourselves with presenting, on the present occasion, the results of the elementary analysis of urinary and fæcal urobilin.

Nevertheless it seemed to us to be necessary to give some account, at this place, of one of the most important of the artificial products referred to, viz.:—that obtained by Maly[1] by the action of sodium amalgam upon bilirubin—because Maly believed that he had obtained by this means a substance identical with urinary urobilin (a view which has met with very wide acceptance), and because this is the only member of the group of which elementary analyses are forthcoming.

Several observers (MacMunn, Thudichum, Disqué, Eichholz) who have repeated Maly's experiments have questioned the identity of hydrobilirubin with urobilin, and Disqué in particular has maintained that the process employed is not capable of yielding a pure product. Disqué[2] and Eichholz[3] have further shown that when the action of

[1] *Centralb. f. d. med. Wissensch.* IX. p. 369. 1871. *Ann. der Chemie u. Pharmacie*, CLXIII. p. 77. 1872.

[2] *Zeitschr. f. physiol. Chemie*, II. p. 259. 1878–79.

[3] This *Journal*, XIV. p. 326. 1893.

sodium amalgam is allowed to proceed beyond the stage indicated by Maly a product is formed which resembles the urinary pigment more closely.

It will appear then that in this connexion two distinct questions present themselves for consideration. In the first place, is the hydrobilirubin which Maly prepared and analysed identical with urobilin? and if not, is it possible to obtain urobilin by the further action of sodium amalgam upon the product?

We have repeated Maly's experiment many times, using in some instances bilirubin which we had ourselves prepared from human gall-stones and from bovine gall-stones (for a supply of which latter we would express our hearty thanks to Dr Lewis Jones), and in other instances with pure bilirubin obtained from Herr Merck of Darmstadt. When the action of sodium amalgam was allowed to proceed to the stage indicated by Maly there was obtained, on acidification with hydrochloric acid, a precipitate of purple flocculi such as he described, and which, when filtered off and purified according to his directions, conformed in its properties to the published descriptions of hydrobilirubin.

It will be obvious to anyone reading Maly's description of his product that if the account of the properties of natural urobilin given in the first part of this paper be correct the two substances are by no means identical. It is true that an acid solution of hydrobilirubin shows the absorption band of acid urobilin and that with zinc chloride and ammonia a brilliant green fluorescence is obtained, but here the close resemblance practically ceases.

Although urobilin is partially precipitated by acids from a concentrated alkaline watery solution it comes down as an impalpable red powder and never in large flocculi as hydrobilirubin does. Again, acid alcoholic solutions of pure urobilin have a yellow colour, passing on to orange and brown on concentration, whereas similar solutions of hydrobilirubin appear dark red or purple. Lastly, although Maly's specimens showed only the single band shifted towards red in alkaline solution, it has been the experience of several more recent observers, ourselves included, that alkaline solutions, and those to which zinc chloride and ammonia have been added, show three bands.

Le Nobel[1] obtained this three-banded spectrum with a specimen of hydrobilirubin which had been prepared by Maly himself, but this

[1] *Arch. f. d. ges. Physiol.* XL. p. 501. 1887.

observation is open to the objection that the substance may have become altered by long keeping.

More important even than such differences as the above is the fact that, as we shall presently point out, combustion analyses of the urinary and fæcal pigments show that hydrobilirubin and natural urobilin differ somewhat widely in their ultimate composition. The most striking difference is in the proportion of nitrogen, and as we shall show a determination of the nitrogen in hydrobilirubin gave us a figure agreeing very closely with that obtained by Maly.

We are thus driven to the conclusion that the first of the two questions above propounded must be answered in a negative sense, and that the hydrobilirubin of Maly, whether it be a definite chemical individual or no, is not identical, either in its properties or in its composition, with natural urobilin.

Passing on to the consideration of the further question we may say at once that the results which we have obtained by allowing the action of sodium amalgam to proceed further agree closely with those of Disqué and Eichholz. As the action proceeds the liquid assumes a pale yellow colour, the extra alkaline bands disappear and the precipitability of the urobilin-like product by hydrochloric acid is conspicuously diminished. When acidified, filtered and exposed to the air the liquid darkens and the absorption band gains in intensity. The product so obtained bears a far closer resemblance to the natural pigment than Maly's hydrobilirubin does.

If an alkaline solution be acidified with acetic acid the urobilin-like substance may be precipitated, as natural urobilin is, by saturation with ammonium sulphate. In alcoholic solution with acetic acid it has a yellow or orange colour and shows a band like that of acid urobilin, with a more intense absorption towards red and a dark shading extending towards the violet.

With ammonia the liquid assumes a pale yellow tint and, as with urobilin, the absorption band disappears. On the addition of sodium or potassium hydrate to alkalinity the band is seen narrowed and displaced towards red, and a similar single displaced band is seen with zinc chloride and ammonia, accompanied by the characteristic green fluorescence.

We have endeavoured to obtain the product in as pure a condition as possible by repeated precipitation with ammonium sulphate and also by extraction with ether-chloroform by the method described in Part I. of this paper, but we have found that, even with the purest solutions

obtained, the tint of the liquid is altered by mineral acids which impart to it a distinct reddish tinge.

The measurements of the several absorption bands above described agreed exactly with those of the urobilin band under like conditions, and absolutely the only spectroscopic differences observed were that with acid solutions the intensity of the band was less than that of the band shown by solutions of urobilin of corresponding depths of colour, and that its redward border was considerably less sharply defined.

On the other hand although we have made repeated attempts, under the most favourable conditions and with the purest specimens which we had at our disposal, to obtain the *E* band spectrum[1] with this artificial product, we have never succeeded in doing so. On the addition of acid to an alkaline aqueous solution partial precipitation of the pigment occurred, but the precipitate was browner than that got with natural urobilin.

Lastly, even the purest solutions do not bear evaporation on the

[1] Since we described the *E* band spectrum in Part I. of this paper (This *Journal*, xx. p. 125. 1896) it has been independently described by a French observer Saillet (*Revue de Médecine*, xvii. p. 109. 1897) who, being unacquainted with our previous account of it, thus independently confirmed our observation. Saillet differs from us, however, in ascribing the development of the *E* band to a chemical rather than to a physical change in the pigment, and bases this opinion upon the fact, which he observed, that upon shaking the liquid showing the *E* band spectrum with acetic ether a pink solution is obtained which shows still a fresh absorption band near the *b* line. We have repeated Saillet's experiment and can in turn confirm his observation.

The substance obtained in solution bears a great resemblance to the modified urobilin which we have described (*loc. cit.* p. 139), and the fact that a modified form of the pigment is so obtained does not appear to us to be incompatible with our view that the *E* band, which the acetic ether solution does not show, is due to a peculiar physical condition of solid urobilin.

We take this opportunity of expressing our regret that when speaking in Section III. of this paper (*loc. cit.* p. 129) of Zawadski's observations upon the action of calomel upon an alkaline solution of urobilin we unwittingly misrepresented his contention. When writing the passage referred to, more than a year after our repetition of his experiments, we were under the impression that Zawadski regarded the pink colour obtained in the alkaline liquid as indicative of the formation of urorosein, whereas it was a product extracted by amylic alcohol after the addition of hydrochloric acid that he believed to be identical with that substance.

In our experiments in which we used natural urobilin, and not the artificial product which Zawadski employed, the *E* band spectrum appeared on the addition of a slight excess of hydrochloric acid and the amylic extract showed only the urobilin band. No product resembling urorosein was obtained.

Our attention was called to this error by reading a recent paper by Salaskine (*Archives des Sc. Biologiques St Petersb.* v. p. 377. 1897) who likewise failed to obtain urorosein by Zawadski's method.

water bath as those of natural urobilin do. Such treatment tends to bring about changes, and on redissolving the residues solutions are obtained which have a red and in some instances even a green colour.

Hence we are led to the conclusion that by the prolonged action of sodium amalgam upon bilirubin a product is formed which presents very remarkable resemblances to natural urobilin but which differs from it in being decidedly less stable.

VIII. *Combustion analyses of Urinary and Fœcal Urobilin.*

In the first part of this paper we described a process for the separation of urobilin from urine which we believed to be capable of yielding the pigment in a condition pure enough to justify its submission to ultimate analysis[1]. By a modification of the preliminary steps this process is rendered available for the extraction of a pure product from fæces also[2].

It will be seen, on reference to our original description, that after a preliminary precipitation of the pigment by a modification of Méhu's method, a procedure is followed which is akin in principle to the well-known process of Stas for the separation of alkaloids from organic mixtures. The pigment is caused to pass from aqueous solution into an organic solvent, and from the latter back again into water, the change of solubility necessary for the production of this alternating transference being brought about, not, as in the Stas process, by a change in the reaction of the solvents, but by the presence or absence respectively of ammonium sulphate. Water containing urobilin will, if saturated with this salt, yield up the pigment on shaking with chloroform or with a mixture of chloroform and ether, whereas in the absence of the salt the water again withdraws the urobilin from the organic solvent.

We believe that the effect of ammonium sulphate upon a solution of urobilin is, in this respect, largely specific, and its application to the separation of this pigment from other urinary or fæcal constituents results in a highly selective process.

To apply the method to the extraction from fæces it is merely necessary to make first an alcoholic extract of the material, to evaporate it to dryness and to extract the residue with distilled water. The

[1] *loc. cit.* p. 120 *b*. [2] *loc. cit.* p. 138.

aqueous solution is then treated exactly as is urine. It is unnecessary to repeat here the general description of the method which was given in the early part of our paper, especially as we have thought it desirable to give an exact account of the preparation of each product analysed in connexion with the results of the analysis. Certain minor modifications of the process were adopted in individual preparations.

The several products submitted to analysis, whether obtained from urine or from fæces, agreed exactly in possessing the spectroscopic and physical properties which were attributed to pure urobilin in the first part of this paper.

The pigment was obtained in each instance as a brown amorphous residue with a characteristic aromatic odour which was shared alike by the fæcal and urinary products. MacMunn has referred to the emission of this peculiar odour as a property of urobilin. All our preparations yielded the *E* band with an ease which is in itself a criterion of purity. One property of pure urobilin not referred to in our earlier account is its remarkably low melting point. The products melted below the temperature of the water-bath and passed on cooling into a brittle shellac-like form which is characteristic. If a moderately strong solution of the pigment be evaporated at the temperature of the water-bath the residue assumes this transparent form and the solid substance examined with the spectroscope shows a broad band at *F*.

Analyses of Urinary Urobilin.

Product 1. Five litres of the urine of a patient with hepatic cirrhosis, which even after great dilution showed a well-marked urobilin band, were saturated with ammonium chloride. The resulting precipitate of ammonium urate was washed with a saturated solution of ammonium chloride until nearly free from adherent urobilin. It was then found to contain a little uroerythrin (as shown by Thudichum's green reaction with caustic alkalies) and it yielded to acid alcohol an extract which showed the bands of acid hæmatoporphyrin faintly.

The filtrate from the urate precipitate was next saturated with pure ammonium sulphate, was rendered faintly acid with sulphuric acid, and then allowed to stand for two days. A bulky precipitate settled and was filtered off. The filtrate was found to contain an abundance of urochrome.

The precipitate was washed with a saturated solution of ammonium sulphate until the washings were colourless, was dried in air and extracted with successive quantities of cold distilled water until no more pigment went into solution. A small quantity of a brown substance was found to be insoluble in the water, and this when dissolved in alcohol showed no distinctive spectroscopic appearances.

The aqueous solution of urobilin was again saturated with ammonium sulphate and the resulting precipitate was then found to be almost completely soluble in water. The second aqueous solution was once more saturated with the ammonium salt, was rendered faintly acid with sulphuric acid and *immediately* shaken with a mixture of chloroform and ether in large separating funnels. Two extractions sufficed for the transfer of all the pigment from the saturated water into the organic solvent, but some slight loss occurred owing to precipitation at the junction of the liquids.

The chloroform-ether was next separated and shaken with distilled water, a few drops of dilute sodium carbonate solution being added to the mixture to reduce its acidity, but not enough to render the fluids alkaline[1]. The whole of the pigment now returned to the water.

This final aqueous solution was once more saturated with ammonium sulphate. One constant effect of the saturation at this stage is to cause a separation of such ether and chloroform as were previously dissolved in the water, and these carry with them a certain amount of the pigment. The thin superficial layer of the organic solvents so produced was, in this instance, allowed to evaporate spontaneously, and the floating residue of urobilin thus left upon the surface, and the precipitate which had meanwhile fallen as the result of the saturation with ammonium sulphate were filtered off together. The filter paper was washed with a neutral saturated solution of ammonium sulphate, was allowed to dry in the air, and was then extracted, in a flask, with strong alcohol. The solution was filtered and evaporated and the residue was redissolved in specially prepared absolute alcohol freshly distilled from sodium.

[1] It may here be mentioned that if the chloroform-ether solution be strongly acid the transference to water is not complete. If, under these circumstances, weak alkali be added drop by drop and the mixture be shaken after each addition, a point will be reached (short of neutrality) at which the transference becomes easy. The water should not be rendered alkaline as it is possible that the process may become less selective than when the pigment both leaves and returns to acidified water.

The product obtained after filtration and evaporation was analysed with the following results.

0·1010 grm. yielded CO_2 0·2305 grm. H_2O 0·0687 grm. [1] Ash = 0·0023 grm.

$$C = 63{\cdot}69\ \%. \qquad H = 7{\cdot}73\ \%.$$

0·188 grm. yielded 6·2 c.c. N at 11·5°C. and 762·5 mm. of mercury

$$N = 4{\cdot}02\ \%.$$

Product 2. Some four litres of urine from a case held to be one of Pernicious Anæmia were submitted to precisely the same processes as were employed in the preparation of the first product. The final product had all the properties of No. 1.

0·02 grm. ignited on platinum left no visible ash.

0·1424 grm. yielded 5·11 c.c. N at 18° and 771 mm. of mercury.

$$N = 4{\cdot}22\ \%.$$

Product 3. This was prepared from the urine of a patient with intestinal obstruction who, for a short period, passed large quantities of urobilin (as judged by the intensity of the absorption band). During this period the patient was under the influence of morphia.

The treatment adopted was in the earlier stages identical with that above described, but in this instance the transfer from water to ether-chloroform was twice repeated and the final stages of the separation were slightly modified. The second ether-chloroform extract (amounting to a bulk of about 1½ litres) was shaken with very weak aqueous ammonia. Some 70 c.c. of this sufficed to withdraw all the pigment and thus great concentration was obtained. A slight excess of sulphuric acid was added to the ammoniacal solution and it was then shaken with pure chloroform which took up most of the urobilin. The chloroform was separated and washed with small quantities of water until nearly neutral (a process which involved some loss of pigment) and was then filtered and evaporated. The residue, after being freed from traces of ammonium sulphate by solution and re-solution in absolute alcohol, was employed for the estimation of nitrogen. 20 mgm. gave no visible ash.

0·206 grm. yielded 7·1 c.c. N at 18°C. and 770 mm. of mercury.

$$N = 4{\cdot}05\ \%.$$

[1] This figure for the ash is probably too high as the boat was visibly contaminated with copper. The product left a little iron.

Product 4. This was prepared from the mixed urines of various hospital patients, for the most part in surgical wards. Each separate quantity obtained was preserved by immediate saturation with ammonium chloride. Only such specimens as showed a well-marked urobilin band were employed.

When, after about a fortnight, seven or eight litres had been collected the mixture was filtered from the urate precipitate produced by the ammonium chloride. The filtrate was then, as usual, saturated with ammonium sulphate.

In preparing this product it was determined to avoid the use of ether-chloroform, and to employ the method of repeated precipitation with ammonium sulphate described in the first part of this paper[1]. The precipitate first obtained was therefore extracted with water until no more pigment was dissolved; to this solution finely powdered ammonium sulphate was added, with brisk stirring until some turbidity appeared. This occurred short of saturation, but before adding more sulphate the solution was filtered[2]. The precipitate thus removed was in this instance very slight and appeared to contain no pigment other than urobilin. The filtrate was next completely saturated and the precipitate of urobilin was filtered off.

The process of precipitation and re-extraction with water were thrice repeated, the final extraction being made with dilute aqueous ammonia, by which means a highly concentrated solution (50—60 c.c. in bulk) was obtained. This was acidified with sulphuric acid, which threw down the urobilin as a reddish powder. The precipitate was removed by the centrifuge, washed two or three times with a neutral saturated solution of ammonium sulphate, again centrifuged and after being freed from any trace of ammonium sulphate by repeated solution in absolute alcohol, was analysed with the following results.

$$0{\cdot}1154 \text{ grm. yielded } CO_2\ 0{\cdot}2676,\ H_2O\ 0{\cdot}0790.$$

There was no weighable ash, but a minute quantity of iron was present.

$$C = 63{\cdot}24\ \%. \quad H = 7{\cdot}60\ \%.$$

0·101 grm. of this product yielded, by Kjeldhal's method, ammonia neutralising 5·9 c.c. $\frac{N}{20}$ acid.

$$N = 4{\cdot}09\ \%.$$

[1] *loc. cit.* p. 118 *a*. [2] *ibid.* p. 135.

Analysis of Fæcal Urobilin.

Product 1. A quantity of the stools of a case of typhoid fever in the early convalescent stage was mixed with a large proportion of methylated spirit and the extract was filtered. The filtrate was taken to dryness at a low temperature and the residue thoroughly extracted with water. The aqueous solution filtered from an abundant insoluble residue was made just acid with sulphuric acid, saturated with ammonium sulphate and allowed to stand. In addition to the brown pulverulent precipitate ordinarily obtained at this stage some peculiar black resinoid material floated on the surface of the saturated liquid. A portion of this dissolved in alcohol showed no absorption band until it was rendered somewhat strongly acid whereupon a well-marked urobilin band developed. The substance was evidently some precursor or compound of urobilin, left undecomposed by the weak acid originally present and precipitated intact by the ammonium sulphate. Possibly it consisted of crude urobilin-calcium as it left a considerable proportion of lime on ignition. The entire precipitate, including this resinoid material, was filtered off and re-extracted with water, the solution being now rendered more strongly acid (up to 2 or 3 $^0/_0$ of sulphuric acid).

Ammonium sulphate now threw down a precipitate free from the resinoid material. This was dissolved in water, passed through ether-chloroform, and subjected to exactly the same final processes as the two first urinary products.

The ultimate product agreed completely in all its properties with the other preparations which have been made, whether fæcal or urinary. It should however be added that in this instance the fæces contained in addition a small proportion of a modified urobilin which had a reddish colour in solution, and showed the general characters of the modified pigment described in the first part of this paper[1].

The modified pigment accompanied that of the typical form through all stages of the process of separation until once transferred to ether-chloroform, when it absolutely refused to leave the organic solvent for water even under conditions of alkalinity.

That portion of urobilin which was present in the ordinary form was transferred from ether-chloroform to water and back again with

[1] *loc. cit.* p. 139.

the accustomed ease; and this portion, which alone formed the material for analysis, had all the characters of urinary urobilin.

0·1936 grm. yielded N 7·2 c.c. at 19° C. and 750 mm. of mercury.

N = 4·17.

Product 2. Normal fæces were extracted with strong spirit. The alcohol was evaporated at a temperature of 40° C. and the residue was thoroughly extracted with water. The bulky aqueous extracts so obtained were saturated with ammonium sulphate, and from this point onwards the material was treated exactly as in the preparation of the first and second products from urine. The final product could not be distinguished as to appearance, odour, spectroscopic properties or general behaviour from the purified pigment of urinary origin. A portion of the product, on a glass dish, examined with the spectroscope showed a band like that of neutral solutions of urobilin.

·1343 grm. yielded CO_2 0·3142 grm.
H_2O 0·0992 „
Ash 0·0009 „ (The ash contained iron)

C = 63·81 %
H = 8·20 „

The Nitrogen of Maly's Hydrobilirubin.

Confronted with the fact that all our analyses of the natural pigment showed a percentage of nitrogen so much smaller than that assigned by Maly to hydrobilirubin, we were anxious to place side by side with our results an estimation of the nitrogen of Maly's product made by ourselves. Accordingly one-third of a gramme of pure bilirubin was reduced with sodium-amalgam, and the product was separated in exact accordance with the original directions of Maly[1]; rather more than a decigramme of hydrobilirubin being ultimately obtained. We thought it better to work with a small quantity of pure bilirubin than with a larger quantity of a pigment the purity of which was less certain. The operation offered no difficulties when carried out on this scale.

0·0998 grm. yielded 8·1 c.c. N at 17 ° C. and 770 mm. of mercury.

N = 9·57 %.

Maly's formula requires N = 9·45 %, and the mean of his experimental results gives N = 9·22 %.

[1] *Annalen d. Chem. u. d. Pharm.* CLXIII. p. 77. 1872.

Summary of Results.

	Urinary Products				Fæcal Products	
	No. 1	No. 2	No. 3	No. 4	No. 1	No. 2
C	63·69	—	—	63·24	—	63·81
H	7·73	—	—	7·60	—	8·20
N	4·02	4·22	4·05	4·09	4·17	—

	Urobilin	Hydrobilirubin		
	Mean of above results	Theory	Mean of Maly's results	Our estimation of nitrogen in hydrobilirubin
C	63·58	64·86	64·68	—
H	7·84	6·75	6·93	—
N	4·11	9·45	9·22	9·57
O	24·47	18·94	19·17	—

Data obtained from analyses of an amorphous substance which has to be first separated from complex mixtures can only be accepted after careful criticism. Nevertheless we have some confidence that the determinations given in this paper will be found worthy of attention.

In the first place we have had in urobilin to deal with a substance which, although amorphous, possesses well-defined individual characters; secondly, we believe that any who will consider the method which we have employed for its separation will recognise that it has the characters of a really selective process; and lastly we may adduce the satisfactory agreement in the figures obtained from products of diverse origins, as affording reasonable evidence that we have worked with a substance of chemical individuality.

It will have been noticed that the urines used for the different preparations were passed under such widely different morbid conditions as hepatic cirrhosis, pernicious anæmia and intestinal obstruction treated by morphia, whilst the fæcal pigment was obtained both from normal and morbid sources.

At the same time it must be admitted that our figures for carbon and hydrogen were obtained from quantities of material somewhat too small for great accuracy, and the analyses leave some uncertainty with regard to the question of ash. Although errors thus introduced must be small and cannot affect our main conclusion as to the fundamental difference between natural urobilin and Maly's hydrobilirubin, we do not feel ourselves in a position to attempt to assign an empirical formula to the former. The figures obtained do not appear to lend themselves

to a formula showing any simple relationship to that accepted for bilirubin, and until experiment has shown by what chemical steps a product strictly agreeing in its general characters with natural urobilin can be prepared from bile pigment it is undesirable to pursue the question of its constitution.

We may be permitted to say that we entered upon the analysis of urobilin obtained from natural sources in the hope that our results might help to place upon a firmer foundation the belief, which has prevailed since the publication of Maly's results, that there exists a *simple* relationship between that pigment and bilirubin. This hope has not been justified by the results, and we are convinced that the relationship is by no means so simple as has been supposed. The change from bilirubin to urobilin cannot be a mere question of reduction and hydrolysis, but must necessarily be attended by a removal of nitrogen; of this our analyses leave no doubt whatever.

On the other hand we cannot doubt that the one pigment is actually derived from the other, a conclusion which evidence of other kinds appears to us to render unavoidable.

It is a well-known fact that in health the bile pigment which enters the duodenum disappears, as such, before the intestinal contents are expelled, and in its place we find in the fæces urobilin and its chromogen.

When, as in certain cases of typhoid fever, the bile pigment is found in abundance in the fæces, the urobilin is greatly diminished in quantity or altogether wanting. When the flow of bile into the intestine is arrested urobilin and its chromogen disappear from the fæces, to reappear when the patency of the bile ducts is re-established.

Friedrich Müller[1] has further shown that when bile is introduced into the stomach of a patient with complete biliary obstruction and whose fæces are urobilin-free, urobilin appears in the stools.

It therefore remains to seek further evidence as to the series of chemical changes which the bile-pigment undergoes in the alimentary canal, and especially on the lines of those experimenters who have succeeded in preparing an artificial product resembling urobilin by the action of intestinal micro-organisms upon bilirubin. Of the product so obtained as well as of various other artificial urobilin-like products we hope to speak in a third instalment of this paper.

[1] *Schlesische Gesellsch. f. vaterland Kultur*, Jan. 1892.

Conclusions from Part II.

1. The hydrobilirubin of Maly although resembling the urobilin of urine and fæces in certain of its properties, differs from it in other respects and notably in containing a far larger proportion of nitrogen.

2. By allowing the action of sodium amalgam upon bilirubin to proceed beyond the stage indicated by Maly a product is obtained which resembles natural urobilin much more closely and which possesses almost identical spectroscopic properties, though we have never obtained from it the *E* band spectrum described in the first part of this paper.

3. In their elementary composition urinary and fæcal urobilin are identical.

4. From analyses of products obtained, from four different supplies of urine and two different supplies of fæcal material, for the most part by modifications of the ether-chloroform process, the following mean figures were obtained for the carbon, hydrogen and nitrogen respectively.

C 63·58, H 7·84, N 4·11.

5. An estimation of the nitrogen in hydrobilirubin yielded a percentage (9·57) which differs but little from that obtained by Maly (9·22).

[The cost of the above investigations was in part defrayed by Grants from the Government Grants Committee of the Royal Society.]